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WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

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REVIEW

In the era of rapid mRNA-based vaccines: Why is there no effective hepatitis C virus vaccine yet?

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Abstract

Hepatitis C virus (HCV) is responsible for no less than 71 million people chronically infected and is one of the most frequent indications for liver transplanta-tion worldwide. Despite direct-acting antiviral therapies fuel optimism in controlling HCV infections, there are several obstacles regarding treatment accessibility and reinfection continues to remain a possibility. Indeed, the majority of new HCV infections in developed countries occur in people who inject drugs and are more plausible to get reinfected. To achieve global epidemic control of this virus the development of an effective prophylactic or therapeutic vaccine becomes a must. The coronavirus disease 19 (COVID-19) pandemic led to auspicious vaccine development against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus, which has renewed interest on fighting HCV epidemic with vaccination. The aim of this review is to highlight the current situation of HCV vaccine candidates designed to prevent and/or to reduce HCV infectious cases and their complications. We will emphasize on some of the crossroads encountered during vaccine development against this insidious virus, together with some key aspects of HCV immunology which have, so far, ham-pered the progress in this area. The main focus will be on nucleic acid-based as well as recombinant viral vector-based vaccine candidates as the most novel vaccine approaches, some of which have been recently and successfully employed for SARS-CoV-2 vaccines. Finally, some ideas will be presented on which methods to explore for the design of live-attenuated vaccines against HCV.

Key Words: Hepatitis C virus; Vaccine candidates; Nucleic acid-based vaccines;

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Core Tip: Hepatitis C virus (HCV) remains a global health burden despite the successful introduction of direct-acting antiviral therapies. In order to achieve global control of HCV epidemic a vaccine is necessary. Its development has faced many hurdles, reason why it is still elusive. Herein, we describe all the challenges during HCV vaccine research, focusing on HCV immunology and emphasizing on current vaccine candidates, particularly nucleic acid-based as well as recombinant vector-based vaccines. We also highlight the impact of severe acute respiratory syndrome coronavirus-2 vaccine race on the renewed interest on HCV vaccine production. Finally, we present ideas on live-attenuated vaccine approaches against HCV.

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INTRODUCTION

Hepatitis C virus infection and the need for a vaccine

Hepatitis C virus (HCV), discovered in 1989[1], represents an important health burden. In 2015, the World Health Organization (WHO) estimated that there were at least 71 million people chronically infected with HCV, which represents a global prevalence of approximately 1%[2]. Additionally, around 400000 deaths occurred from infection complications.

Infections with HCV cause both acute as well as chronic liver disease in 60%-80% of the cases. Chronicity is associated with the development of cirrhosis (15%-30%) and hepatocellular carcinoma (HCC)[3]. Liver damage resulting from this infection makes it one of the most frequent indications for liver transplantation worldwide[4-8].

The problem of HCV infections worldwide has led the WHO to propose the elimination of viral hepatitis as a public health burden by 2030[2]. However, in order to achieve this goal, big scale interventions are needed, such as screening testing, effective treatment and hopefully vaccination, the latter still non-existing for HCV.

Access to widely available screening tests is uncommon and is hindered by economic reasons, particularly given the fact that new HCV infections are mainly asymptomatic[9]. This leads to an underestimation of the disease prevalence and does not contribute to the eradication goal. Concerning treatment, the development of interferon-free (IFN-free) regimens based in direct-acting antivirals (DAAs) has revolutionized HCV therapy. These antivirals have significantly increased response rates (up to 98%) and greatly reduced treatment duration to only 8-12 wk of oral treatment. DAAs have generated optimism on the global control front, and some consider that this pathogen can now be effectively controlled solely by means of antiviral therapy[10,11]. However, there are some limitations and obstacles to keep the virus in check, in particular, the cost and practical aspects of treatment access, which is uneven among different countries and leaves underdeveloped regions without treatment[11]. Additionally, resistance to DAAs emerged concomitantly with their development and implementation. Resistance-associated substitutions have been detected both before as well as during and after treatment with DAAs[12]. Another interesting aspect to consider is that eliminating HCV infection with DAAs does not eradicate the risk of developing liver cancer. Also, protective immunity is usually insufficient after natural or treatment-induced viral clearance, thus, the possibility of reinfection remains[13]. Together, these facts make HCV elimination in high-risk groups a very challenging task and the need for an effective prophylactic vaccine remains the greatest uncovered medical problem in the hepatitis C field[14]. Vaccination against HCV infection would reduce public healthcare resources by avoiding expensive DAA-based regimens or medical treatments for any liver or metabolic

complications derived from long-term infections[15-17], especially in low- or middle-income countries, where HCV prevalence is still moderate-high and access to diagnosis and treatment uneven and costly[18].

Proper immune responses are able to clear HCV acute infections, preventing the progression to chronicity (in 20%-40% of infected individuals). This fact suggests that vaccination could be a reasonable goal[19] provided we grasp a better understanding of immune responses against HCV in order to develop different vaccine candidates that allow for appropriate protection.

Global epidemic control will only be possible if the number of new HCV infections is reduced alongside with an increased number of cured patients[11,14]. However, a recent report showed that almost 60% of 91 surveyed countries had, in 2016, higher rates of infection than cures, making the goal of HCV elimination as a health burden by 2030, difficult to achieve[20].

For all the reasons previously mentioned, safe and effective prophylactic and/or therapeutic vaccines are necessary for the global control of HCV epidemic[11,21-24]. Indeed, no infectious disease has been controlled and eradicated with antimicrobial treatment, while it has in fact been possible by vaccination[10]. Furthermore, effective vaccination strategies widely available have been the only unfailing method to keep viral transmission at bay by providing herd immunity[25]. Modelling studies have indicated that, even with the introduction of new DAA treatments, only a quasi-eradication of HCV would be possible[26,27], highlighting the need for a vaccine against HCV.

Two extraordinary and unique situations that took place during this last year have fueled optimism on vaccine development against HCV. First, the Nobel Prize in Physiology or Medicine 2020 for the discovery of HCV which was awarded last October [28]. Three distinguished researchers, Harvey J. Alter, Michael Houghton and Charles M. Rice, received the prize for their contribution in identifying the etiological agent of the hepatitis formerly known as non-A non-B, and enabling the development of screening tests and antiviral drugs for its treatment. All of them expressed their hopes for a future vaccine against hepatitis C in their Nobel lectures, and Charles M. Rice specifically stated that he hoped we can learn from all the efforts that are being put into developing coronavirus disease 19 (COVID-19) vaccines[29]. This last state-ment refers to the second event from last year that has renewed interest on HCV vaccines: The COVID-19 pandemic and the remarkable development of several vac-cines to fight it. In the same line, in June 2020, the National Institutes of Health (NIH) opened a grant opportunity for the design of vaccines against HCV assigning USD 8 million to this aim[30].

This review focuses on different vaccine candidates designed to prevent or diminish HCV infection cases, and summarizes all the pitfalls encountered during vaccine development against this virus, including some key aspects of HCV immunology. We make special emphasis on nucleic acid-based vaccines as well as recombinant viral vectors and provide information on severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) vaccines as examples of approaches that might be important in HCV vaccine development.

Prophylactic vs therapeutic vaccines

Vaccine candidates with two different goals have been considered to control HCV epidemic: Prophylactic and therapeutic (primary and secondary prevention, respectively). The most widespread use of vaccination has always been to prevent a particular disease (prophylactic vaccination)[31] by building immunity in an individual prior to the first encounter with the pathogen, and thus becoming immune to a particular illness. On the other hand, therapeutic vaccination is meant to induce immune responses against a disease that is already in course in a given individual[32].

As we will later discuss in detail, the challenges for designing an effective prophylactic vaccine are vast (HCV variability and diversity, limited animal models and a complex immunological response). Many preventive vaccines against other viral patho-gens are able to induce neutralizing antibodies (nAbs) that correlate with protection, which seems to be difficult to achieve for HCV[14]. Nevertheless, even a low efficacy prophylactic vaccine might be useful to decrease the epidemic impact in high-risk populations by reducing the number of new infections[33-35].

Therapeutic vaccines against HCV have great potential to aid in controlling chronic infections by increasing curing rates or reducing therapy duration[36]. In this new DAA era, sustained virological response (SVR) rates are extremely high (above 98%) and treatment duration has already been shortened compared to classic dual therapy (pegylated IFN-α plus ribavirin). However, there are difficult to treat patients (with active HCC or severe liver decompensation, those experiencing multiple DAA

treatment failures, or those infected with HCV genotype 3)[37] for which this therapeutic approach would be beneficial. These vaccines would boost HCV-specific T cell responses and would help in three different ways: (1) Preventing viral relapse if therapeutic vaccines were to be administered in conjunction with DAA therapy; (2) Maximizing early viral clearance and thus increasing SVR rates by first employing a therapeutic vaccine followed by the antiviral treatment; and (3) Producing partial control of HCV infection just by means of therapeutic immunization and thus reducing viral load[38]. Despite promising results in decreasing viral titers, rebounds have been observed, most likely due, either to immune escape or the inability of properly inhibiting viral replication or eliminating most of HCV-infected hepatocytes[21].

Expected outcome of effective vaccine candidates

In general, effective vaccine candidates should stimulate generation of nAbs and a proper cellular immune response. In order to design vaccines that elicit protective immunity against HCV, it is of utmost importance to consider the virus tropism (mainly hepatocytes), transmission route (parenteral transmission through contaminated blood) and pathogenesis[39].

A vaccine that induces immune responses similar to those produced by individuals which have successfully cleared the virus after an acute HCV infection, might prove valuable[19]. As we will discuss in the next section, vigorous responses of broadly cross-reactive CD4+, CD8+ T cells to conserved epitopes[40-42], as well as nAbs contribute to HCV spontaneous clearance[43,44].

ADAPTIVE IMMUNE RESPONSE IN HCV INFECTION

Approximately 20%–40% of HCV-infected patients clear the virus spontaneously, while the rest develop a persistent infection that will result in severe fibrosis, cirrhosis and HCC[3,45]. Thus, it is essential to understand the immune protection induced during acute infections in patients that achieved spontaneous viral clearance in order to determine the immune parameters that a successful vaccine has to reach.

Multiple evidences in human and animal models have demonstrated the undoubted association of spontaneous viral clearance with a broad, sustained HCV-specific T cell-mediated immunity (CMI) to conserved HCV non-structural proteins[46,47] and nAb targeting conserved regions of viral envelope glycoproteins E1E2[48].

As will be detailed below, both arms of the immune response are primed during HCV infection, but the characteristics vary depending on whether an acute infection is spontaneously resolved or if it evolves to chronicity.

Cellular immune protection

While HCV-specific CD8+ T cells are the main effector cells, the outcome of infection depends on eliciting efficient virus-specific CD4+ T cell responses[49]. These cells are the central regulators of adaptative immunity providing help for priming CD8+ T cell response as well as antibody response during viral infections. The breadth of the T cell response is a key determinant to spontaneously clear HCV. High numbers of CD4+ and CD8+ T cells targeting different epitopes were observed in individuals who resolved acute infections in comparison to those who evolve to chronicity[42,50,51]. These cells are multi-specifically targeting both structural and non-structural HCV proteins[46,52,53]. However, CD8+ T cells targeting non-structural proteins are immunodominant and associate with spontaneous clearance[54].

The strength of the CMI is also important for HCV infection outcome. Indeed, a robust HCV-specific CD8+ T cell response is associated with the resolution of acute HCV infection[55]. In an acute infection, cytotoxic T lymphocytes (CTLs) have cytolytic and noncytolytic functions which mediate viral eradication[56]. They traffic from the lymph nodes to the liver, where they recognize HCV-antigenic peptides loaded on human leukocyte antigen class I in infected hepatocytes. These infected cells can be lysed through the action of perforins and granzymes, or, killed *via* Fas/FasL interactions that activate the caspase cascade and end up in the apoptosis of the target cell. The noncytolytic function occurs without destroying infected cells, where viral replication is inhibited by cytokines released by CTLs which generate an antiviral environment.

Broad specific CD4+ T cells are detected during the acute phase regardless of the final outcome. However, these cells undergo an early decrease in frequency and breadth in persistent HCV infection compared to patients who clear the infection spontaneously[57]. Thus, spontaneous resolution is associated with a CD4+ T cell

response significatively stronger in comparison to persistently, or chronically infected individuals[58,59].

In chronic infections, the limited functionality of specific CD4+ T cells due to the lack of proliferative capacity and cytokines production[59-61] leads to a dysregulated CD8+ T cell response which facilitates the emergence of escape viral variants[62]. Dysfunctional CD8+ T cells are unable to control the viral load and become exhausted because of the persistent exposure to HCV epitopes which have not mutated[63]. Thus, these exhausted T cells undergo a progressive loss of their cytotoxic activity, proliferative capacity and proinflammatory cytokines production[64,65]. However, it is of note, that the cytolytic activity, and in particular the Fas/FasL dependent function, are associated with HCV immunopathology. Fas expression is up-regulated in hepatocytes of an infected liver whereas FasL is expressed in CTLs. This leads to liver damage by apoptosis of both infected and bystander hepatocytes, and subsequent liver fibrosis development[66].

Humoral immune protection

During acute HCV infection antibodies are produced and target epitopes in both structural and non-structural proteins, however, the envelope glycoproteins E1 and E2 are the main targets of the humoral immune response. Located at the N-terminal end of E2, the hypervariable region 1 (HVR1) is an immunodominant motif[67], which is the most variable region of the HCV genome[68]. Mutation in neutralizing epitopes allow the virus to escape from isolate-specific nAbs[69-71].

Early studies reported that nAbs developed against HCV target the HVR1 region of E2, however these nAbs were isolate-specific[67,69]. Thus, diverse studies have identified monoclonal antibodies (mAbs) that target conserved sites across multiple HCV genotypes located on either linear[72,73] or conformational[74,75] epitopes on E2 ectodomain.

Analyzing sera from different patients who were infected with the same HCV isolate showed that 43% of those who resolved their infections had nAbs against the main HVR1 variant, whereas these antibodies were present only in 13% of patients who evolved to chronicity[76]. Interestingly, plasma isolated from HCV-infected patients immediately prior to clearance has a better capacity to neutralize HCV strains from different genotypes compared to acute infection plasma from patients who subsequently evolve to persistence[77,78]. Furthermore, analysis from patients who cleared HCV infection showed detectable level of nAbs at earlier time points in comparison with acute infections that proceed to chronicity[79]. Chronic infections have been associated with a delayed cross-reactive nAbs response[43,77,78,80]. Although cross-reactive nAbs elicited during chronicity are not able to clear the infection, these have been associated with reduced liver fibrosis[81].

Despite the high genetic diversity of HCV, it was possible to isolate broadly neutralizing human Abs (bNAbs) from HCV-infected individuals, capable of neutralizing diverse HCV genotypes targeting relatively conserved regions on envelope glycoproteins[48,75,82]. These bNAbs have shown to be protective against infection in animal models of HCV[75] and are capable of abrogating established HCV infection in a humanized transgenic mouse model[48]. These findings underscore the protective role of the antibody response.

Evidence of protective immunity against HCV reinfection

The resolution of the initial HCV infection does not lead to sterilizing immunity so patients who previously controlled the primary HCV infection can be infected again [83]. However, differential rates of reinfection and/or chronicity have been reported among people who inject drugs (PWIDs) with the same risk of exposure, being reduced in people previously infected in comparison with people without previous infection[84]. Resolution is achieved in about 80% of HCV-reinfected patients[85].

Reinfection was characterized by a significant reduction in duration and magnitude of viremia compared with the primary infection and it was also shown to protect against persistence[85]. Moreover, clearance of reinfection was associated with an earlier and higher frequency of broadened T cells secreting IFN-γ as compared to primary infection[86-89] and an early induction of nAbs[85,90].

Long-lived memory HCV-specific CD4+ and CD8+ T cells are detected in the peripheral blood in humans following spontaneous resolution of the primary infection for up to 20 years[89,91]. CD4+ T cell depletion before reinfection leads to viral persistence even in the presence of functional CD8+ T cells which evidences the protective role of memory T cells upon re-exposure to HCV. While CD8+ T cells are the main effector cells in viral control, CD4+ T cells are essential for CD8+ T cell function and prevent viral escape within epitopes targeted by CD8+ T cells.

CHALLENGES FOR DEVELOPING ANTI-HCV VACCINES

A number of difficulties have hindered the development of vaccines against HCV throughout the years (Figure 1). Despite all the knowledge acquired on the biology of this virus in recent years, a full understanding of key aspects of its pathogenesis and the host's immune response remains elusive. Taking into account the correlate of protection, an effective vaccine needs to be able to prime both arms of the adaptative immune response. Thus, vaccination has to induce an early and sustained expansion of specific CD4+ and CD8+ T cell response. Alongside cellular immunity, cross-reactive nAbs need to be elicited to provide protection against different variants and genotypes.

In this section we will go over the most important challenges on the design and validation of an effective vaccine against HCV.

Lack of economic incentive

Despite the fact that vaccines are great tools to prevent diseases, usually they are not as profitable as are drugs and other health services, and therefore investing in vaccine development is less appealing for the pharmaceutical industry[92]. Additionally, the development of vaccines with two different aims (prophylactic and therapeutic) would probably be expensive, and including prime/boost vaccination strategies may result impractical[19]. On another front, most newly infected individuals are PWID which mainly belong to populations with limited financial resources. This represents another discouraging aspect for companies interested in vaccine development[19].

From an economic perspective, though, there is well-reported evidence that vaccines are, in the long run, the most cost-effective public health measure after access to clean water[93,94]. A vaccine to fight HCV will, most likely, not be an exception.

Viral genetic diversity and variability

HCV is an enveloped virus with a single-stranded positive RNA genome which has a single open reading frame (ORF) flanked by non-coding regions at both ends (5′ and 3′). For these features, it is classified as the prototype member of the Hepacivirus genus within the *Flaviviridae* family[95]. The ORF codes for a polyprotein of around 3000 amino acids which is co- and post-translationally processed into three structural (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B)[96].

Mutation is a key mechanism contributing to HCV genetic diversity and it is mainly driven by the error prone NS5B RNA-dependent RNA-polymerase[97]. HCV has an approximate mutation rate of 10^{-5} mutations/nucleotide/replicative cycle[98,99], a characteristic which together with big population sizes, short generation times, and high replication rates generates the intra-host circulation of a complex population of closely related genome variants, usually termed as viral quasispecies[100,101]. Of utmost importance is the N-terminus of the envelope protein E2[67]. It contains the HVR1 region of about 30 amino acids which exhibits a huge variation among different isolates, and it is the most variable region of the entire HCV genome[68]. Even though most HCV-infected individuals develop nAbs against the virus, this high variability represents a problem as it allows the virus to escape immunologic surveillance and prevents the development of vaccines that induce cross-reactive nAbs[21]. Thus, a major challenge for the development of a broadly reactive vaccine for the control of HCV infection is identifying conserved neutralizing epitopes outside of HVR1.

Notably, mutations within HVR1 have also been associated with resistance to cross-neutralizing antibody response even if their epitopes are conserved, which highlights again the difficulties in achieving HCV neutralization as HCV could persist even in the presence of an antibody response to conserved epitopes[102,103]. This finding suggests that the neutralizing capacity of an antibody should not only consider the degree of conservation of its epitope.

Mutation rates coupled with the selective pressure exerted by the host's immune system has steered HCV diversification into 8 genotypes and 90 subtypes[104,105]. HCV strains from different genotypes differ by 30% in their nucleotide positions within the coding region, whereas subtypes exhibit 15% nucleotide variation[106]. Genotypes 1 and 3 are the most prevalent worldwide (accounting for 49.1% and 17.9% of diagnosed cases, respectively), and are most frequently found in developed countries[107].

The quasispecies dynamic as well as the resulting viral diversity confers HCV an amazing ability to adapt which in turn implies the possibility to escape from different therapeutic or preventive approaches such as antiviral drugs or vaccines[108-112].

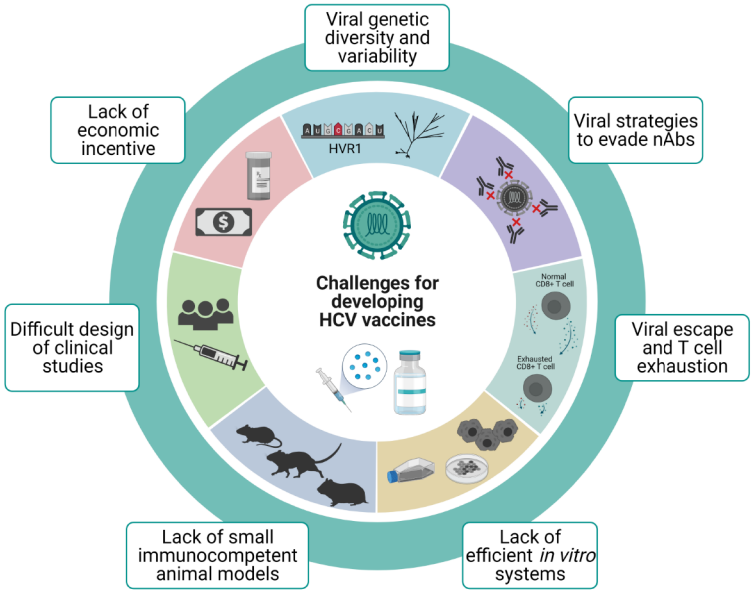

Figure 1 Challenges in hepatitis C virus vaccine research. Graphical representation of all the hurdles yet to be overcome in order to develop effective vaccines against hepatitis C virus. Image created with BioRender.com. HCV: Hepatitis C virus. HVR1: Hypervariable region 1.

Thus, T cell-based vaccines intended to induce broadly reactive immune responses by targeting more conserved regions/proteins of the virus are desirable if the aim is to protect against new infections and/or persistence[11,21].

Viral strategies to evade neutralization by antibodies

Viral entry to host cells and viral interactions with different host factors could theoretically be blocked by nAbs targeting HCV envelope glycoproteins E1 and E2. However, the virus has evolved several mechanisms which affect the host´s ability to neutralize the virus. One of the mechanisms has been described extensively above (genetic diversity, particularly in HVR1 region), yet there are a number of other strategies employed by this virus to evade neutralization: (1) Glycosylation of structural proteins; (2) Cell-to-cell transmission; (3) Interfering antibodies; (4) Association with lipoproteins; (5) Antibody decoy; (6) Flexible conformational epitopes; and (7) Enhancing of viral entry.

Glycosylation of structural proteins: This feature reduces their immunogenicity as they are recognized as selfstructures. This is an important mechanism used by HCV to escape host humoral immune response. Glycans act by masking antigenic sites targeted by nAbs, interfering sterically with antibody neutralization[113]. Indeed, the deletion of N-glycans leads to an increase in E1E2 immunogenicity and can induce a more potent antibody response against HCV[114-116]. Glycan shift is another mechanism to induce neutralization resistance through glycosylation. Single point mutations which result in deleting a glyco-sylation site or generating a new glycosylation site in another part of the protein could facilitate viral resistance to neutralization. It has been reported that a new glycosylation site arose after incubating for 5 d a cell-culture derived HCV with nAbs obtained from mice. As a result, those broadly nAbs showed a decrease in their efficacy[117].

Cell-to-cell transmission: It is another mechanism for viral dissemination, which avoids the extracellular compartment and favors escaping host humoral immune responses[118,119].

Interfering antibodies: When non-nAb bind to sequences in the C-terminal region of HVR1, they disrupt the recognition of conserved epitopes by antibodies with neutralizing capability. Indeed, the remotion of interfering antibodies in chronic patients and vaccinated chimpanzees increases virus susceptibility to neutralization highlighting the role of interfering antibody in viral escape[120]. Similarly, when HVR1 was removed, enhanced and broad cross-neutralizing activity was observed[121,122].

Association with lipoproteins: HCV circulates in the blood in association with triglyceride-rich lipoproteins and low-density lipoproteins forming hybrid lipoviral

particles, which are a hallmark of infectious HCV particles. Several host-derived factors play a role in evading antibody neutralization. Lipoproteins such as apolipoprotein E contribute to humoral immune escape by hiding relevant neutralization epitopes in E2 protein, preventing them to be exposed during HCV assembly and maturation, hence, abrogating antibody neutralization[123,124].

Antibody decoy: Interestingly, *in vitro* studies have reported that HCV-infected cells release E2-containing exosomes that act as antibody bait making HCV virions less susceptible to neutralization[125].

Flexible conformational epitopes: The capacity of some conserved neutralizing epitopes in E2 to adopt different conformations when complexed with diverse antibodi-es contributes to evade neutralization by antibodies. This conformational flexibility must be taken into account during vaccine design[126].

Enhancing of viral entry: It has been shown that host mutations that alter the interaction of serum components like high-density lipoprotein with scavenger receptor BI enhance viral entry to the cell[127]. This, in turn, protects the virus against humoral response as the time window in which nAbs can bind and act is reduced[128,129]. Fofana *et al*[130] (2012) also showed that mutations in the E2 glycoprotein, conferred viral escape to humoral responses by altering the use of the T cell receptor CD81[130].

Despite these challenges, it has been possible to isolate broadly cross-neutralizing mAbs with the ability to block HCV infection of various genotypes and thus, protect against heterologous viral infection[75,131-134]. These findings suggest that a prophylactic vaccine against HCV may indeed be achievable.

The elucidation of the crystal structure of E2 has provided a better insight into different antigenic domains and regions that allow a rational vaccine design. A study showed that epitopes within E2, exhibiting moderate or conserved variability, were efficiently targeted by bNAbs[135,136]. Unfortunately, despite the relative conservation of some bNAbs epitopes, escape mutations have been identified[137,138].

Escape mechanisms from T cell responses: Viral escape and T cell exhaustion

Several studies have evidenced the key role of cellular immunity in the clearance of infection. An effective vaccine has to induce a rapid recall of the memory T cell responses that is associated with reduced viraemia and a higher likelihood of spontaneous resolution. However, the virus has developed different mechanisms to lead to an inefficient cellular response even when re-exposed with homologous virus: (1) Viral escape T cell recognition; and (2) T cell exhaustion.

(1) Escape mutations within major histocompatibility (MHC) class I-restricted HCV epitopes represent the main mechanism used by HCV to evade CTL responses and thus it is associated with persistence. Unlike CD8+ epitopes, escape mutations within targeted CD4+ T cell epitopes are not common, suggesting that CD4+ T cells failure mechanisms cannot be completely explained by viral escape[139]. Escape mutations occur early in infection and they are rare during long-term chronic infection, possibly due to the lack of T cell-mediated selective pressure[140]. Interestingly, escape variants show an impaired replicative fitness[141,142] and this contributes to limiting the variability within some epitopes[143,144]. As a consequence, the ideal target for T cell-based vaccines are conserved epitopes less likely to mutate because of viral fitness cost [141,142]. Another effect of escape variants results in impaired recognition by T cells receptors and thus prevents CD8+ T cell recognition. Moreover, CD8+ T cells from infected patients with genotype 4 were not able to recognize epitopes from other genotypes[52]. This finding highlights the challenging task of choosing vaccine targets that protect against multiple HCV genotypes. Hence, identifying conserved epitopes recognizable by specific CD8+ T cells is a key point to develop efficient T cell-based vaccines.

(2) T cell exhaustion: While T cell-based vaccines likely provide protection against chronic virus infections, they also have the potential to generate immunopathology following subsequent virus infection. This is illustrated by the fact that during chronic infection an impaired HCV-specific CD8+ T cell response develops, known as T cell exhaustion. This phenotype is associated with the inability of the immune system to control viraemia during chronic infection. These exhausted T cells undergo a progressive loss of their ability to proliferate, to secrete cytokines (such as IFN-γ), and to be cytotoxic[64,65].

Long-lived memory T cell response is only induced following spontaneous clearance and it can provide some protection. However, individuals who cannot maintain such long-lived memory T cell response due to T cell exhaustion are not

protected upon re-exposure.

One of the major challenges for immunogenic T cell vaccines refers to the recovery of T cell immunity through vaccination in people with persistent HCV infection. Kelly *et al*[145] (2016) demonstrated that when an HCV T cell vaccine based on chimpanzee adenoviruses (ChAd3) are given to patients with chronic disease, the immune response is not able to restore T cell function[145]. Failure to respond to this vaccine approach may be the result of T cell exhaustion, as vaccination is stimulating memory responses that were induced early in infection but that ended up partially dysfunctional following viral exposure[145].

Lack of efficient in vitro systems

An essential step in vaccine research is the evaluation of antibodies generated as a result of natural infections or experimental immunizations, as well as the evaluation of vaccine candidates. For those purposes using different *in vitro* and animal models becomes a must[23].

As we will exemplify in a later section on vaccines against SARS-CoV-2, the generation of live-attenuated and/or inactivated whole virus vaccines has been possible against a number of different viruses (measles, mumps, rubella, rotavirus, hepatitis A virus, poliovirus, among others), however this strategy is not achievable to generate HCV vaccines. Since HCV was discovered[1], and only until recently, research has been thwarted by the inability to culture the virus both *in vitro* and *in vivo*[23,146].

As for *in vitro* models, propagating HCV in cultured cells remained limited for several years since inoculation of patient sera or plasma in different cell lines resulted in limited or no viral replication[147]. The first report of efficient replication came from working with HCV subgenomic replicons (where the structural region was replaced by a neomycin-encoding gene)[148]. However, the challenge was to generate an *in vitro* system that was able to produce infectious HCV particles at high titers that would allow further research[23]. The production of cell-culture derived viral particles (HCVcc) was only achieved in 2003 with the discovery of a genotype 2a isolate (strain JFH-1) derived from a Japanese patient with a fulminant hepatitis[149,150]. Transfecting replicon HCV RNA from isolate JFH-1 into human hepatoma-derived Huh7 cells resulted in efficient RNA replication without the need of any adaptive mutations[150,151]. Nevertheless, despite this breakthrough, efforts to replicate this with other isolates corresponding to different genotypes were only partially succe-ssful. On the one hand, some of these cloned full-length RNAs were able to produce infection *in vivo* (in chimpanzees), but on the other hand, even in the presence of multiple adaptive mutations, they failed to produce infectious viral particles in cell culture, despite some being able to efficiently replicate (details on the history of HCV cell culture systems are thoroughly reviewed elsewhere[147,152-154]).

Further studies on HCVcc led to the discovery of more permissive cell clones derived from Huh7 cells (*e.g.*, Huh7.5 and Huh7.5.1)[155,156] as well as to the generation of inter- and intragenotypic recombinant genomes that are able to recapitulate the complete HCV life cycle and produce high titers of infectious particles *in vitro*. These recombinants have been shown to be optimal *in vitro* models to study the neutralization ability both of mAbs as well as of sera from infected patients[82,157-160]. They have also been used to characterize antibody escape mutations[71,137,161]. Additionally, reporter and flag-tagged JFH-1-based genomes (J6/JFH1) have been generated[162-164] and used in vaccine development[165], the latter in particular to facilitate large-scale purification of viral particles[163]. However, the most important aim in this field would be to efficiently grow any virus derived from HCV infected patients, which unfortunately has not yet been achieved[153]. For now, we depend on the constructs described above as well as a few full-length consensus clones, which have been developed after a lot of research effort and had to be designed including numerous adaptive mutations[166-170], therefore, not quite resembling natural circulating isolates. In spite of the setbacks, all these constructs have the potential to be employed for producing inactivated whole-virus vaccines.

Another *in vitro* approach to assess the neutralizing ability of sera and mAbs, in addition to HCVcc, relies on the generation of HCV pseudoparticles (HCVpp). These are generated by cotransfecting HCV E1 and E2 genes together with a retroviral packing and reporter system[171]. Due to the struggles imposed by the generation of different HCVcc derived viral particles, HCVpps were actually developed earlier[172,173] but continue to be used in vaccine research nowadays[157,174-176].

Lack of small immunocompetent animal models

Humans are the natural hosts of HCV, and in order to test the efficacy and safety of vaccine candidates in pre-clinical studies, *in vivo* animal models are needed. Foremost, *in vivo* studies on pathogenesis of HCV chronic infections have been problematic since HCV only infects humans and, under experimental conditions, also chimpanzees. The first and most successful immunocompetent animal model has indeed been the chimpanzee. However, ethical concerns and its inclusion on the United States Fish and Wildlife Service's Endangered Species have led to a ban in its use for biomedical research[177]. Even before this prohibition, the continued use of these animals faced many issues such as high costs, small cohort sizes which made statistically significant results difficult to achieve, and the inability to genetically manipulate chimpanzees. Furthermore, it would require the need to have special and expensive facilities to breed and keep them under study[178].

Small animal models are frequently very useful tools to test potential vaccine candidates, but, since HCV does not infect rodents, a lot of effort has been devoted into developing strategies to adapt mice to evaluate HCV vaccines. This led to the use of chimeric humanized or transgenic mice with humanized livers[179] or expressing human CD81 and occludin[180], two cellular proteins that HCV uses as receptors for cell entry. However, mouse models are difficult to produce, and most are immunocompromised, which makes them inappropriate to study virus-host interactions and immune responses. Additionally, they do not exhibit cirrhosis or HCC[181]. In spite of this, genetically humanized fully immunocompetent inbred mice expressing human orthologs of HCV entry factors were developed[182], which have allowed the study of viral entry, yet not the full viral cycle. To address the latter, Chen *et al*[183] (2014), developed an immune-competent humanized mice model that is capable of developing persistent HCV infections and hepatopathological manifestations[183], yet the mice stock are outbred and genetically not well defined. More recently, Keng *et al*[184] (2016) were able to establish a new humanized mouse model including human hepatocytes as well as human immune system[184], which was able to recapitulate HCV infection and immunopathogenesis[181], although low levels of B cells were detected when compared to clinical settings.

For the difficulties in getting broad access to small immunocompetent mouse models, alternative experimental non-human primate models have been explored. However, no signs of infection were detected (for a detailed review see Ploss and Kapoor[178], 2020), with the exception of tree shrews (now classified in a separate order Scandentia, but previously designated as small squirrel-like primates) which can become symptomatic and even progress to chronicity[185]. Despite this encouraging finding, keeping these animals in captivity is a difficult task, and additionally they are genetically diverse for being an outbred species, which again poses issues to be widely used in HCV biomedical research[178].

Altogether, this shows us the difficulty we face when we need animals that can be employed for vaccine development but also to study HCV-associated pathogenesis. An alternative could be the use of substitutes and analogue viral models that can be propagated in mice lab strains and that appear to share basic immunological features with HCV. Recently, the discovery of non-primate hepaciviruses has raised interest since they can be used as analogues of HCV infection[23]. A rodent Hepacivirus discovered in Norway rats[186] has been shown to establish high-titer liver infections when inoculated in immunocompetent mice, and thus, provides insight into hepatic immune responses[187]. However, the main drawback of this model is the limited sequence homology to HCV[186]. On the other hand, equine hepacivirus (eqHV), formerly known as non-primate Hepacivirus, is the closest relative of HCV and both species share some important features such as the level of E1E2 glycosylation or the presence of miR-122 seed sites in their 5′ non-coding regions (2 sites in HCV and 1 site in eqHV)[188,189]. These approaches of using alternative and analogue viral models for vaccine development is extremely valuable, yet it is worth acknowledging that different mammalian immune systems might respond in different ways and this should be taken into consideration at the moment of interpreting data[23].

Difficulty in designing clinical studies

The design of clinical studies for HCV vaccine candidates poses its own hurdles. It must be considered that, in order for an effective vaccine to be validated, it should be tested in populations at risk for HCV infection[11,36]. This is an issue in developed countries where HCV infection incidence is low other than in PWID populations. Targeting this group of patients has ethical concerns and practical difficulties to be overcome[190]. Despite this, there are a few studies which have been successful in

identifying, enrolling and monitoring PWID before developing an acute HCV infection [191,192], the latest completed phase I/II clinical trial with outcome results was able to enroll 548 active intravenous drug users (ClinicalTrials.gov Identifier: NCT01436357 [193])[194]. On the other hand, large studies could be conducted where incidence is higher, such as some developing countries. However, logistical problems may arise due to the large number of patients needed and their appropriate follow up, specifically to detect acute cases of hepatitis, which usually course without any symptoms[36].

APPROACHES TO DESIGN VACCINE CANDIDATES FOR HCV

There are several traditional and newer approaches in vaccine development, and most of them have been explored for the design of HCV vaccine candidates (Figure 2), albeit the majority only directed at genotype 1.

Traditional vaccine approaches include whole-organisms vaccines containing either inactivated whole or live attenuated viruses. Live attenuated vaccines are potent in inducing CMI and humoral immunity and have been successful for many viral infections because they resemble what occurs naturally. Nevertheless, they have the potential risk of reverting to virulent wild-type strains. In contrast, inactivated viruses are noninfectious but have the downside of being less immunogenic than attenuated viruses. Therefore, when inactivated whole viruses are developed as vaccine candidates, they often include adjuvants and/or booster injections in order to enhance the immunogenicity[195].

Newer methods involve the use of one or more genes of the virus of interest to be incorporated into the genome of a nonpathogenic organism for amplification. In this way, mainly three different approaches have been developed: Subunits vaccines (by purifying the protein/s of interest generated in the heterologous organisms), DNA vaccines (usually by isolating a plasmid containing the gene/s of interest), and recombinant viruses (by using the entire host virus as a live vector)[195].

The latest method successfully explored has been the use of RNA-based vaccines, whose development is faster than other technologies, easily scalable, and of lower cost to manufacture. These characteristics have been essential to the development and recent authorization for emergency use of some of the vaccines currently available to control the COVID-19 pandemic[196].

In this section we will go over some of the vaccine candidates explored against HCV, and we will delve into nucleic acid-based and recombinant viral vector approaches.

Inactivated whole virus (HCVcc)

This traditional approach of inactivated virus was only feasible after the development of cell culture systems, with all the challenges that they impose even nowadays. This is partly the reason why there are only a few pre-clinical studies assessing the immunogenicity of inactivated HCVcc as vaccine candidates[197,198]. Both studies have shown the induction of humoral immune responses in chimeric mice[198] as well as in a non-human primate model[197]. The latter also elicited T cell responses. These findings are promising, but there are still some developmental challenges to overcome if this approach is to be considered for clinical trials, such as production in serum-free culture conditions and scalable and cost-efficient downstream processes. Fortunately, there are a few studies which have addressed these difficulties, and have shown that high titer serum-free HCVcc is possible for different intra and intergenotypic recombinants based on JFH-1 isolate[199] and that more efficient downstream processes based on ultracentrifugation and chromatography can be applied[200]. Nevertheless, the challenge of generating high titers of HCVcc of the most widespread genotypes and subtypes still remains.

Recombinant subunits and synthetic peptides

Recombinant E1/E2 proteins were the first prophylactic vaccine candidates being tested since they are the major targets for nAb, in particular HVR1 region within E2. They were shown to be able to induce the generation of nAb in chimpanzees[201], yet only one candidate reached clinical trials in 2007 (ClinicalTrials.gov Identifier: NCT00500747[202]). Results of the phase I trial in healthy volunteers showed the vaccine was well-tolerated at different doses used, and that it was able to induce antibody production[203,204].

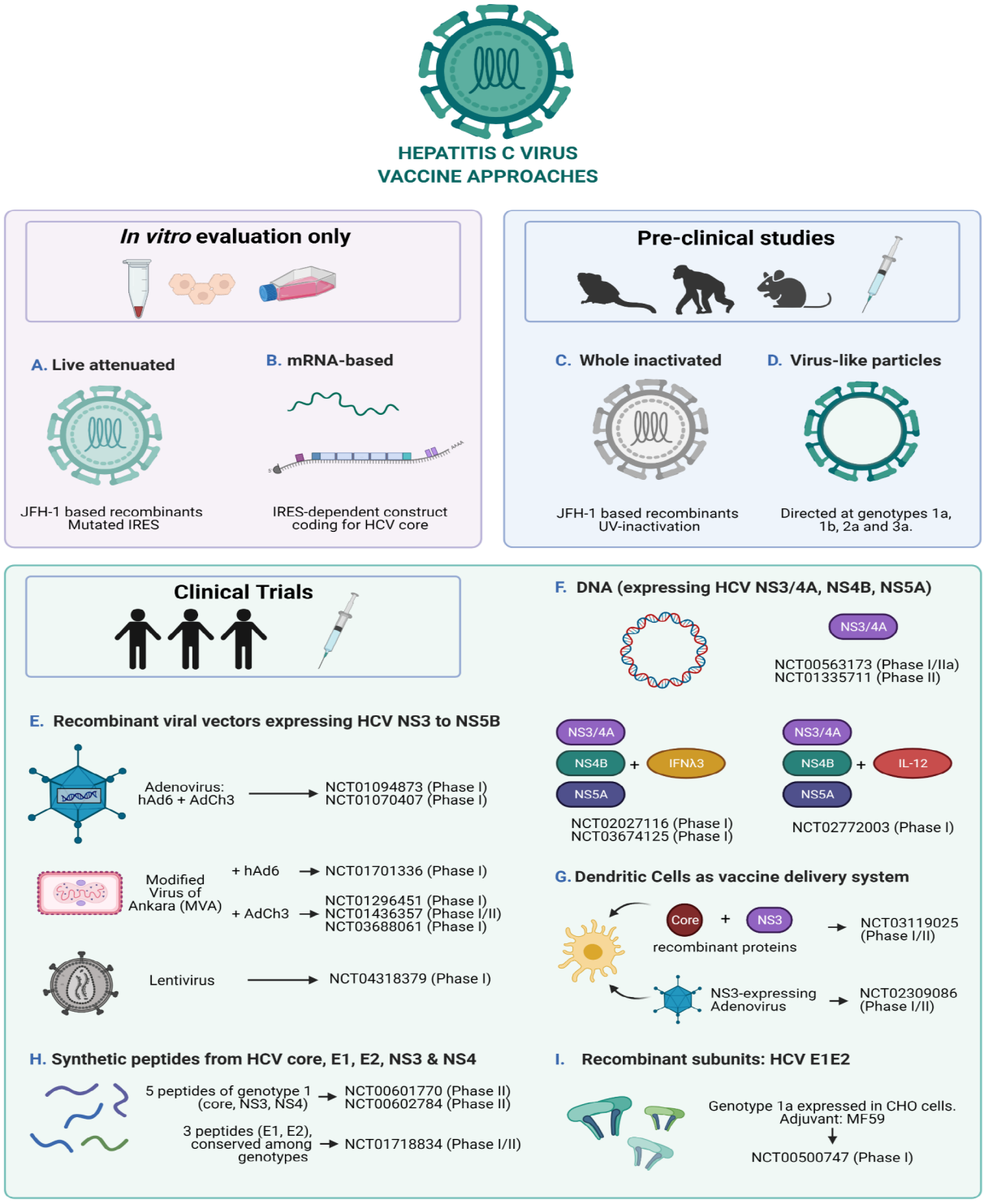

Figure 2 Summary of all hepatitis C virus vaccine approaches explored to date. The studies are divided in three categories depending on the highest stage of research achieved: *In vitro* evaluation only (in lilac background), pre-clinical studies in different animal models (in light blue background) and clinical trials in healthy volunteers and/or chronically hepatitis C virus-infected patients (in green background). For each approach (A to I) key characteristics on the vaccine candidates are provided. In addition, for all the technologies that have reached clinical trials, the ClinicalTrials.gov Identifier and the phase of the trial are indicated. Image created with BioRender.com.

Whereas recombinant E1E2 vaccines were designed to elicit humoral immune response, synthetic peptide vaccines are more attractive since they can be designed to prime both arms of the immune response. Some peptide combinations targeting both cytotoxic lymphocytes and CD4+ T cell epitopes (core, NS3, NS4) have entered clinical trials. Results for the phase 2 trial NCT00602784[205] have shown that the peptide vaccine IC41 can trigger T cell responses in relapse patients after dual therapy, yet viral clearance was not achieved[206]. Unfortunately, humoral response was not analyzed. The results of the other studies remain to be published (ClinicalTrials.gov

Identifier: NCT01718834[207] and NCT00601770[208]).

Of interest, computational identification of B and T cell epitopes has been explored as an alternative for the rational design of effective vaccine candidates. By means of different immune-bioinformatic and population dynamics simulation approaches, many predicted epitopes in E2, NS3/4A, NS5A and NS5B have been identified[209-212]. These approaches provided valuable information and *in silico* screening methods for highly conserved immunogen candidates with the putative ability to block escape mutations (for a detailed review please see[213]). These computational designs can help speed up vaccine development at the experimental stages by rationally selecting the most promising epitopes for subunit vaccine *in vitro* and *ex vivo* evaluation.

Virus-like particles

Virus-like particles (VLPs) are particles that resemble a virion but do not contain the viral genome, rather they are generated by the auto assembly of structural proteins in a manner that is genome-independent. In this way, the particle is similar to the native virus but it lacks the ability to replicate and for vaccine candidates is a very attractive technology since they are more immunogenic than soluble proteins and can prime both arms of the immune response[214].

The rationale behind this type of vaccines is supported by the successful development of vaccines against hepatitis B virus and human papilloma virus, currently commercially available[23]. Unfortunately, despite having shown promising pre-clinical results[215,216], to the best of our knowledge, HCV VLPs have not yet reached clinical trials.

Recombinant vector-based vaccines

The use of live recombinant viral-based HCV vaccines as a genetic immunization approach has shown to be powerful for eliciting CMI[217]. For this purpose, different modified viruses are used as vectors to carry HCV genetic information[19].

Adenoviral vectors are the most widespread used in the vaccine developing industry. They are attractive models for different reasons: Adenoviral genomes are well characterized and are relatively easy to modify into replication-defective viruses, most human adenoviruses cause mild infections, they infect a broad number of cell types (dividing and non-dividing), they can be grown to high titers in tissue culture, and by deleting essential genes, genetic information of interest can be inserted[218]. The most frequently used in immunization studies is the human adenovirus serotype 5 (hAd5), which is included in at least 12 of the vaccines against SARS-CoV-2 that are currently on clinical trials and in one that already had authorization for emergency use (Sputnik V vaccine)[219,220]. Despite their benefits, individuals might exhibit preexisting anti hAd5 Abs, which could diminish the immune response to vaccines based on this viral vector. For this reason, less frequent serotypes such as hAd24, hAd6 or hAd26 have been employed in pre-clinical and clinical studies of vaccine candidates against different viruses[221-223]. Additionally, adenoviruses that infect chimpanzees (AdCh3) have been tested in conjunction with hAd6, both carrying HCV non-structural proteins NS3 to NS5B of genotype 1b, yet despite reaching clinical trials, they have only been evaluated in phase I studies (ClinicalTrials.gov Identifiers: NCT01094873[224] and NCT01070407[225]). The reason for not continuing these studies seemed to be the inability to restore CMI, and as a result, a non-significant effect on HCV viral load was observed[145].

In light of these drawbacks, another viral vector has been employed in prime/boost vaccination strategies against HCV: The Modified Virus of Ankara (MVA), an attenuated poxvirus strain which is immunogenic and safe since it lacks several immunomodulatory genes[226]. MVA vector together with hAd6, both expressing HCV non-structural proteins NS3 to NS5B have entered phase I clinical trials to evaluate the combination as a therapeutic vaccine to be used in conjunction with dual therapy (ClinicalTrials.gov Identifier: NCT01701336[227]). Even though the study is complete, no results have been disclosed, presumably due to the newer DAA treatments which have completely substituted classical therapy. The most promising trials currently in phase I and II use the combination of ChAd and MVA vectors harboring HCV NS3-NS5B genomic regions. A phase I study in healthy volunteers showed promising results in terms of eliciting T cell responses (ClinicalTrials.gov Identifier: NCT01296451[228])[229]. Unfortunately, a phase I/II study in PWID population showed that this vaccination strategy was not effective for preventing chronic infections since T cell exhaustion was not reversed (ClinicalTrials.gov Identifier: NCT01436357[193])[194,230]. These results highlight the need for a vaccine strategy that stimulates both humoral and T cell immunity[23,231]. However, attempts to enhance CMI without the need of boosting the generation of Abs, have been

addressed in pre-clinical studies on non-human primates by fusing the HCV non-structural antigen to MHC class II-associated invariant chain[232]. The results showed enhanced and accelerated CD8+ T cell responses and paved the way to reach clinical trials. At the time of writing this manuscript, there is an actively recruiting phase I clinical trial (ClinicalTrials.gov Identifier: NCT03688061[233]) that seeks to enroll 25 healthy participants to assess the safety and immunogenicity of HCV prime/boost vaccination with both ChAd and MVA vectors expressing HCV non-structural antigens fused to a class II-invariant gene. Results from only 15 individuals seem promising, largely mimicking pre-clinical studies, but more participants are still needed and assessment of durability of the enhanced CMI needs to be further addressed[234].

The most recent vector-based therapeutic vaccine candidate entering phase I clinical trials is a lentiviral based HCV immunotherapy (HCVax) which aims to evaluate both the safety and the immune response in chronic HCV patients (ClinicalTrials.gov Identifier: NCT04318379[235]). Last generation lentiviral vectors are safer than first generation ones (previously used for gene therapy) and like adenoviral vectors, are capable of infecting both dividing and nondividing cells, and since they integrate into the host's genome, expression of the transgene can be long-term, a characteristic which makes them attractive as vaccine strategy[236].

Nucleic acid-based vaccines

Nucleic acid based-vaccines present numerous advantages over traditional vaccine approaches: (1) No issues associated with misfolding of proteins in recombinant protein vaccines or with high manufacture costs; (2) No infectious risks that might be associated with live-attenuated or inactivated whole virus vaccines; (3) They are able to activate both arms of the immune response (humoral and cellular); (4) The expression of antigens resembles natural epitopes; (5) In a single injection, multiple genes can be delivered; and (6) If multiple doses are needed, unlike the use of recombinant virus-based vaccines, there is no risk of anti-vector immunity[39,237,238].

DNA-based vaccines have been in the picture for nearly 40 years now[239]. They usually consist of purified plasmids which harbor sequences of interest that are expressed under the control of a eukaryotic promoter for a robust expression in mammalian cells. They are inexpensive, easy to manufacture, and also important, stable at room temperature. All of which are features that make them an ideal technology in vaccine research, as distribution and access could be granted effortlessly even to developing countries[39].

RNA vaccines have been explored for around 25 years, beginning with studies of self-amplifying RNA vectors (modified RNA from viruses) as well as mRNA pulsed into dendritic cells (DCs)[240,241], and have been largely assessed for tumor vaccination[242]. They share some features with DNA vaccines, but they do not need to enter the nucleus to translate the genetic information into antigen proteins, which represents an advantage over DNA immunization since the barrier of the nuclear envelope is removed, and thus, their efficacy is higher[238]. However, RNA is more labile than DNA, which might yield less robust vaccines than DNA-based formulations due to RNA shorter shelf life, reason why modified nucleosides have been used to enhance stability and therefore induce a higher antigen production[238], as it is the case of the COVID-19 mRNA Vaccine (nucleoside modified)[243].

The first approach for delivery of nucleic acid-based vaccines, was direct injection of naked DNA plasmid or mRNA (transdermally or intramuscularly), however, efficiency seemed to be very low, in part due to the negative charge of these molecules. There-fore, several delivery methods were developed to improve uptake and immunogenicity in different organisms: (1) Gene gun: DNA is loaded on the surface of tungsten or gold particles and then fired at target cells; (2) Electroporation: Transient pores in cell membranes are created by electrical impulses allowing DNA delivery inside the cell; and (3) Nanoparticles: Non-viral vectors made up from lipids, inorganic molecules and polymers can safely carry DNA and RNA into a cell by encapsulating the negatively charged nucleic acid, preventing its digestion by endonucleases and facilitating intracellular release[36,238].

DNA-based vaccines

Multiple pre-clinical studies in different animal models have been performed throughout the years to assess the efficiency of several DNA-based formulations against HCV to elicit immune responses. Nevertheless, only a few have entered phase I or II clinical trials.

The use of core as antigen, directly injected as naked DNA plasmid intramuscularly (IM) or intraperitoneally (IP) into different mice models, has evidenced a weak immunogenic capacity in terms of humoral response but strong CMI, even though at least 2 doses 2-4 wk apart were administered[244-248]. Using the same delivery method and injection scheme, HCV core and E2 sequences were fused to immunogenic proteins (hepatitis B surface antigen or gD protein from herpes simplex virus type-1) to address the weak Ab response, and both arms of the immune response were detected in mice as well as in rats[249-251]. Others have attempted to evaluate if different localizations of HCV antigens within the cell and the CpG content of the plasmid backbone might influence the Ab response. Results indicated that membrane-bound and secreted E2 forms as well as the addition of immunostimulatory CpG motifs elicited a better humoral response in mice[252]. Low doses of IFN-α have also shown to augment CTL response after DNA immunization with a plasmid encoding HCV core protein in mice models[253].

Targeting structural proteins in DNA-based formulations employing injection of naked plasmid as the delivery method was thoroughly tested in animal models but the vast majority failed to enter clinical trials. With the increasing knowledge on immune correlates during acute infections, it became clear that non-structural proteins are the target of CMI during acute resolutions, and that other delivery methods such as electroporation or gene gun rendered broadly reactive CTL responses[254].

As a consequence, DNA-based vaccines encoding HCV non-structural proteins have become widely used approaches. Transdermal gene gun injection of DNA plasmid encoding NS3/4A proteins into mice has shown high titers of Abs and the ability to prime CD4+ T helper cells[255] and also a CD8+ T cells that were able to clear HCV protein-expressing hepatocytes and persist up to 12-18 mo after immunization[256, 257]. When NS3 DNA vaccine was co-administered with interleukin-12 as adjuvant, strong immunogenicity was also displayed in murine models[258]. Several other adjuvants have also been employed in NS3-based DNA vaccination in order to enhance their potency (for a detailed review see Sepulveda-Crespo *et al*[231] 2020). In addition, constructs encoding a codon-optimized NS5A injected IM into mice, in combination with *in vivo* electroporation, were also able to prime specific T cell responses[259]. Two clinical trials in chronic HCV patients (naïve to treatment, infected with genotype 1) have entered phase I/IIa and phase II to evaluate a potential therapeutic vaccine based on a plasmid encoding NS3/4A (ChronVac-C) (ClinicalTrials.gov Identifier: NCT00563173[260] and NCT01335711[261]). Results have shown that high doses of ChronVac-C were able to activate HCV-specific T cell responses which led to a transient reduction in viral loads[262]. When 8 of the 12 patients enro-lled also received dual therapy after the vaccine doses, 6 were able to achieve SVR, which might indicate that immunization had a beneficial effect on the response to therapy. However, these results seem irrelevant at present with the advent of DAA treatments.

Even though pre-clinical results were promising, full-length NS3 protein exhibits immunosuppressive effects and it is possibly involved in the development of HCC due to its enzymatic activity which deregulates the normal functions of the host cells[263]. Even though DNA immunization renders antigen expression only transiently, and the adverse effects possibly caused by NS3 enzymatic activity would be marginal, alternative plasmids for DNA vaccination encoding modified NS3 sequences have been tested in animal models. Ratnoglik *et al*[264] (2014) showed that vaccinating mice with a non-enzymatic version of NS3 (with its catalytic site and NTPase/RNA helicase domains mutated to abrogate their functions) induced strong CMI, indicating that mutations in this protein do not seem to interfere with its immunogenicity[264]. Additionally, a plasmid with a truncated form of NS3, only encoding immunogenic epitopes (1095–1379 amino acids positions), succeeded in eliciting a strong Ab response after repeated intra-dermal inoculation in mice[265]. However, none of these candidates has reached clinical trials.

These findings seem to indicate that immunizing only with DNA-based formulations coding for NS3/4A or NS5A might not be sufficient to control viremia in HCV-infected patients, despite encouraging pre-clinical results in animal models.

In addition to NS3/4A or NS5A plasmid vaccination, IM injections followed by electroporation of constructs encoding NS3 to NS5B into *Rhesus macaques* and chimpanzees, in multiple-dose boosting schemes, evidenced HCV-specific effector CD4+ and CD8+ T cells and effector memory-like CTLs after immunization[266,267]. More recently, studies in mice have shown that adding a plasmid expressing cytokine IFN-λ3 (formerly known as IL28B) to the immunization with plasmids expressing NS3/4A, NS4b and NS5A provided significant immunoadjuvant activity[268]. These encouraging results led to a phase I clinical trial to evaluate the safety, tolerability and immunogenicity of this strategy in chronic hepatitis C patients infected with HCV

genotypes 1a or 1b, which had previously exhibited treatment failure to dual therapy alone or in combination with DAAs (ClinicalTrials.gov Identifier: NCT02027116[269]). The vaccination strategy comprised a combination of 3 plasmids each encoding NS3/4A, NS4B or NS5A (formerly known as VGX-6150) and a fourth plasmid encoding IFN-λ3 as an efficacy enhancer (the mixture of 4 plasmids has been renamed to GLS-6150). Three different doses were tested in a prime-vaccination scheme of 4 doses every 4 wk, and then a booster immunization at week 36, all injected IM followed by electroporation. Results of this trial have been recently published and they showed that GLS-6150 is safe and was overall well tolerated with no serious adverse events identified[270]. More importantly, vaccination increased the HCV-specific T cell responses, although, surprisingly, RNA viral titers did not decrease. Therefore, considering the reinfection possibility of patients who achieved SVR after DAA treatment, a new phase I clinical trial is ongoing in order to assess immunogenicity of GLS-6150 in this population and in healthy volunteers (ClinicalTrials.gov Identifier: NCT03674125[271]). Another clinical trial employing DNA vaccination of plasmids encoding NS3 to NS5A (INO-8000) but with the co-administration of a different adjuvant (interleukin-12) is currently active as a phase I study in chronically HCV infected patients (genotype 1) (ClinicalTrials.gov Identifier: NCT02772003[272]) which highlights the potential of these approaches including immunostimulatory molecules as adjuvants. The main takeaway of these approaches is that, the addition of more nonstructural genes as well as the co-administration of immunostimulatory adjuvants, might still be insufficient to clear an established infection. The question remains if they might be useful to prevent reinfections.

Therefore, as an alternative, heterologous prime/boost vaccination strategies have also been explored in mice, in which immunization with DNA-based vaccines is followed by immunization with viral vectors such as MVA to enhance response levels [273]. Even though results provided proof-of-concept that 2 different HCV vaccine technologies can improve immunogenicity when used in combination, to the best of our knowledge, so far, no clinical trial has tested this approach.

RNA-based vaccines

As will be detailed in the section about vaccines against SARS-CoV-2, several mRNAs-based vaccine candidates have been intensely explored in clinical trials, in particular to fight the COVID-19 pandemic. However, so far none have been approved for human use, with the exception of some of the vaccines currently in phase 3 clinical trials which are undergoing assessment for WHO emergency use listing and prequalification[274-277] (ClinicalTrials.gov Identifier: NCT04368728[278] and NCT04713553 [279]–Pfizer/BioNTech SE, ClinicalTrials.gov Identifier: NCT04470427[280] and NCT04649151[281]–Moderna TX, Inc).

On the contrary, with the exception of using mRNA to transfect DCs (which will be discussed in the next section), there have been no pre-clinical or clinical trials using mRNA-based vaccines against HCV. Interestingly, Sharifnia *et al*[282] (2019) have proposed for the first time that an RNA-based vaccine against HCV could be feasible since after *in vitro* generation of an mRNA coding for the core protein, they were able to detect core protein in monocyte-derived DCs which were previously transfected with this construct[282]. Unfortunately, no further animal studies were performed to assess the immunogenicity of this approach.

DCs as vaccine delivery system

DCs are one of the most potent antigen-presenting cells needed to induce and maintain immune responses. Given their fundamental roles, DC-based vaccination strategies have been given special attention, in particular for cancer immunotherapy [283]. However, different approaches have also been explored in HCV vaccination both in pre-clinical studies as well as in clinical trials[284]. Strategies involve loading DCs with HCV core, NS3 or NS5 proteins[285,286], pulsing them with HCVpp[287], transfecting them with DNA[288] or mRNA[289], or transducing them with adenoviral vectors expressing HCV non-structural proteins[290-293].

Two recently concluded phase I/II clinical trials have enrolled chronically HCV-infected patients (HCV genotype 1b) to evaluate the safety and clinical efficacy of therapeutic vaccination using autologous DCs. Despite employing different strategies (autologous DCs loaded with recombinant HCV core and NS3 proteins *vs* transduced with a recombinant adenovirus encoding NS3), both studies revealed similar results in terms of immunogenicity and ability to reduce viral titers: T cell responses were generated albeit weakly, and these were insufficient to clear the virus or reduce viral loads[286,293] (ClinicalTrials.gov Identifier: NCT03119025[294] and NCT02309086[295]). These findings are somewhat discouraging since in order to design better

vaccination strategies, attention will have to be placed on enhancing CMI so as to, at least partially, reduce viral titers.

IS THERE A POTENTIAL USE OF ATTENUATED VIRUSES AS VACCINE CANDIDATES AGAINST HCV?

As with whole inactivated virus vaccines against HCV, the limited *in vitro* culture systems have hampered studies on attenuated vaccines. In particular, attenuation has been achieved by serial passaging of a given virus in non-primate cells, which leads to the emergence of mutations that have low fitness in human cells. Yet HCV does not replicate efficiently in non-human cells, which poses problems for the identification and production of attenuated strains. Additionally, there is also the risk of causing an infection after the use of these types of vaccines, which in principle, limits their potential use[11,14]. However, it is worth noting that live-attenuated viral vaccines are licensed for human use for prevention of several viral diseases such as dengue, hepatitis A, measles, mumps, varicella, yellow fever and gastrointestinal disorders caused by rotaviruses[296]. Therefore, if properly designed, this technology offers safe and effective vaccines.

Considering the issue of identifying attenuating mutations in non-human cultures, an alternative is to detect mutations occurring naturally within the human host, present only as minority variants within the quasispecies, and exhibiting an attenuated phenotype.

HCV, as many members of the *Flaviviridae* family (all except for those within the Flavivirus genus), translate its polyprotein in a CAP-independent manner by recruiting the ribosome directly to the internal ribosome entry site (IRES), which is found in the 5′ non-coding region[297]. IRES structure and sequence are essential to its function, and any change can affect the translation process[298,299]. Therefore, investigating on mutations that might affect this process may enable an alternative approach for the design of live-attenuated vaccines against HCV. In this regard, our group has identified several mutations within the IRES of HCV isolates from chronically infected patients of genotype 1a and 3a, that are present in very low frequencies within the viral population, and that have evidenced a significant decrease in viral translation efficiency *in vitro*[300]. Studies in cell culture, using full-genome chimera replicons based on JFH-1 strain are underway in order to assess both translation efficiency as well as viral fitness.

It is important to mention, that one of the initial vaccines designed to fight polio was a formulation with poliovirus (PV) strains where, through successive passages in non-human cells, mutations were selected along the whole genome[301]. Of those, a mutation within PV IRES which drastically diminishes the translation efficiency, is the main responsible for the attenuated phenotype[302]. Unfortunately, live-attenuated PV vaccines have shown to be genetically unstable, and some of the mutations that confer the attenuated phenotype can reverse during replication in humans, causing rare cases of vaccine-associated paralytic poliomyelitis[303]. Thus, if the aim were to design a safe live-attenuated HCV vaccine with mutations in the IRES region, perhaps additional approaches would need to be considered so as to minimize the chances for reversion or enhancing the resulting immune response. One such approach could be constructing a bicistronic vector co-expressing an antiviral protein (for example IFN-β), which has already been proven effective to limit viral spread and to induce antiviral immunity in animal models when assessing a Flavivirus vaccine candidate[304].

On the other hand, a rational synthetic design of attenuated strains might be a new and achievable approach to employ based on the newest infectious replicons that harbor almost the entire genome sequence from non-JFH-1 strains, covering in this way most of the circulating HCV genotypes. This strategy has been successfully developed and tested in mice for other RNA viruses such as Influenza A virus and Coxsackievirus[305]. It consisted of engineering codons that were more prone to generate a Stop mutation after a single nucleotide change in as many positions as possible, without changing the amino acid identity. This strategy proved that the synthetic and rational generation of self-limiting vaccines is possible in different RNA viruses and thus, could represent an alternative way of generating HCV attenuated vaccines as well, provided that the issues with *in vitro* scaling-up production can be overcome in the near future.

LESSONS LEARNT FROM ANTI-SARS-CoV-2 VACCINES

COVID-19, caused by the SARS-CoV-2[306], has become a major health concern all over the world and has spawned challenges to develop safe and effective antiviral drugs and vaccines for preventive use. Vaccine development is a complex and time-consuming process, that typically requires years of research and testing before reaching the clinic. But in 2020, in an unprecedented effort due to the synergy between academia, researchers, and pharmacists, added to financial support and guided by cumulative knowledge from many years of scientific work, scientists were able to produce safe and effective coronavirus vaccines in record time[307]. Coronavirus vaccine types include inactivated vaccines, nucleic acid vaccines, adenovirus vector - based vaccines, and recombinant subunits vaccines. Up until February 18th researchers were testing 70 vaccine candidates in clinical trials, and 20 have reached the final stages of testing. Over 10 have been approved for emergency use in several countries around the word. Among these, it seems important to highlight the Emergency Use Authorization for 2 highly effective mRNA COVID-19 vaccines from Pfizer-BioNTech and Moderna. This is the first time that mRNA-based vaccines have ever been approved for human use, and marks a critical milestone for achievement in both science and public health[275,308,309]. As previously mentioned, mRNA vaccines trigger immune responses by transfecting synthetic mRNA encoding viral antigens (in this case spike protein or protein motifs) into human cells. Once the nucleic acid enters the cytosol of the cell, the mRNA vaccine temporarily induces the cell to produce specific viral antigens coded by the mRNA[308,310]. The major breakthroughs of these two vaccines were: (1) The mRNA modifications and purification process to reduce the innate immune response and to improve mRNA stability; and (2) The effective intracellular delivery to facilitate cellular uptake of mRNA and to protect it from RNase degradation.

These RNA vaccines generate powerful antibody responses to the SARS-CoV-2 coronavirus, but they have not proven to be as good as the AstraZeneca/Oxford vaccine (adenoviral vector vaccine) at stimulating $CD8^+$ T cells. Recently animal studies suggest that a combination of an RNA coronavirus vaccine and a adenoviral vector vaccine (AstraZeneca/Oxford vaccine) could strengthen immune response by rousing $CD8^+$ T cells in mice better than either vaccine alone[311,312]. This preliminary data should be confirmed in upcoming clinical trials.

Thus, what can we learn about SARS-CoV-2 impressive vaccine development? Firstly, that when there is interest and resources, the development and production times of a vaccine can be significantly reduced. Secondly, that mRNA vaccines have a high potency, ability for rapid development, and cost-efficient production. Thirdly, that preliminary data suggests that mixing COVID vaccines technologies boosts the immune response at a cellular level.

Is it possible, therefore, to apply all the knowledge gained from COVID-19 vaccines to accelerate HCV vaccine development? Unfortunately, only partially. As mentioned in the section about challenges, many hurdles remain since HCV biology and immunology differ greatly from that of SARS-CoV-2. However, the so far unexplored possibility of an HCV mRNA-based vaccine could certainly benefit from the experiences and developments in the field of RNA-based vaccines against SARS-CoV-2.

CONCLUSION

HCV is an insidious virus, which, since its discovery, has caused enormous difficulty to be kept under control. The successful introduction of DAAs has become a milestone in keeping the epidemic in line, however it has proven to be insufficient to achieve global eradication of this virus and all the health complications derived from the infection. Therefore, numerous approaches have been explored in order to design an effective vaccine, either prophylactic or therapeutic. Unfortunately, to date, none of these attempts have rendered a viable vaccine for human use. Several drawbacks have hampered its development, among which, to our understanding, one of the most difficult to override is T cell exhaustion, the main cause of therapeutic vaccines failure. However, many other challenges related to a still incomplete understanding of HCV immunology remain to be overcome. Noteworthy among these, is the insufficiency of CMI to control infections and the need for a joint humoral response, as well as the necessity for characterization of better epitopes for nAbs. An approach that might prove effective in the future, is the use of heterologous prime/boost vaccination,

where two different technologies can be employed to enhance the immune responses. Additionally, we believe that ongoing efforts to develop improved and more suitable *in vivo* systems should be a priority, since many of the successful pre-clinical studies have possibly failed in clinical trials due to the differences in immunopathology between the used animal models and humans. All of the hard work that has enabled the rapid and effective development of vaccines against SARS-CoV-2 should be taken as an example of what can be achieved if the interest and the efforts are focused on tackling a health burden. In particular, the advances on mRNA-based vaccine technology, which so far has not been explored in HCV vaccine candidates, would be a good starting point if the aim is to explore alternatives not investigated so far. Additionally, different methodologies which have been shown to be efficacious against other RNA viruses, are available for the design of live-attenuated strains as vaccines against HCV. Following this line of thought, and likely fueled both by the success of COVID-19 vaccines[313] and by the Nobel Prize in Physiology or Medicine 2020 (awarded to three scientists for the discovery of HCV)[28], last year, the NIH opened a grant opportunity for projects concerning HCV vaccine design[30]. As a result, it is expected that more research will be focused on this subject in the upcoming years, and hopefully, auspicious findings will follow. This renewed interest in funding HCV vaccines might be what is needed to achieve HCV global eradication, as has been proposed by the WHO a few years ago. Allocating funds for this purpose boosts the research area that has been left behind in terms of breakthroughs that can be effectively translated to public health benefits.

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